

Stem cell therapy for neonatal hypoxic-ischemic encephalopathy

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Treatments for neonatal hypoxic-ischemic encephalopathy (HIE) have been limited. The aim of this paper is to offer translational research guidance on stem cell therapy for neonatal HIE by examining clinically relevant animal models, practical stem cell sources, safety and efficacy of endpoint assays, as well as a general understanding of modes of action of this cellular therapy. In order to do so, we discuss the clinical manifestations of HIE, highlighting its overlapping pathologies with stroke and providing insights on the potential of cell therapy currently investigated in stroke, for HIE. To this end, we draw guidance from recommendations outlined in stem cell therapeutics as an emerging paradigm for stroke or STEPS, which have been recently modified to Baby STEPS to cater for the “neonatal” symptoms of HIE. These guidelines recognized that neonatal HIE exhibit distinct disease symptoms from adult stroke in need of an innovative translational approach that facilitates the entry of cell therapy in the clinic. Finally, new information about recent clinical trials and insights into combination therapy are provided with the vision that stem cell therapy may benefit from available treatments, such as hypothermia, already being tested in children diagnosed with HIE.

Keywords: cerebral palsy, stem cells, hypothermia, combination therapy, translational research

CLINICAL MANIFESTATIONS OF NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

Hypoxic-ischemic encephalopathy (HIE), cerebral palsy (CP), and periventricular leukomalacia (PVL) are mainly triggered by neonatal hypoxic-ischemic brain injury. Neurodevelopmental deficits such as learning disabilities, mental retardation, and hearing and visual impairments accompany children diagnosed with hypoxic-ischemic brain injury. Brain expression of systemic asphyxia characterizes HIE (1). Perinatal asphyxia and resulting hypoxic-ischemic encephalopathy (HIE) occur in 1–3 per 1000 births in the United States (2). Worldwide, 10–60% of infants who develop HIE will die and at least 25% of the survivors will have long-term neurodevelopmental sequelae (2). Hypoxic-ischemic encephalopathy is the primary cause of 15–28% of cerebral palsy among children (2). Throughout the paper, the terms HIE and the alternative term neonatal encephalopathy (NE) (3, 4) are synonymous. These two terminologies have been a topic of much debate (5, 6). Even with an intense effort by researchers and clinicians to employ precise diagnostic methods, encephalopathy has not been identified in premature infants as compared to full term infants (7–9). HIE brings a relatively high 50% mortality rate in newborns (10), and a small portion of those survivors, 25% display CP symptoms permanently (11, 12). Ischemic perinatal stroke is responsible for 30% of children with CP (13). A cerebral white matter injury, known as PVL, is detected in 50% of neonates with exceedingly low

birth weights with 90% of survivors displaying CP symptoms (14); however, studies using ultrasonography report findings of the incidence of PVL to be lower than 50% (15–17). As a result of the very similar pathophysiological symptoms between neonatal hypoxic-ischemic brain injury and adult stroke, innovative treatments such as cell-based therapies, which are currently being tested in stroke, may prove to be successful in neonatal hypoxic-ischemic brain injury. Having a grasp of the neurochemical cascade of events is a holy grail for commencing treatment intervention in neonates (18). To this end, therapeutic benefits may be achieved by abrogating the “secondary energy failure” or “excite-oxidative cascade” (18, 19). This is characterized by amplified excitation of NMDA receptors combined with peculiar oxidative stress due to mitochondrial dysfunction, altogether depleting energy from the brain seen in infants with hypoxic-ischemic injury (18). Currently, hypothermia is used to treat HIE (20–22) and has demonstrated to be very effective in newborns with a gestational age of ≥ 36 weeks (22, 23) diagnosed with moderate to severe HIE (21, 22), but neurodevelopmental deficits persist in 40–50% of patients even after hypothermia (22). A treatment that combines both hypothermia and cell transplantation may prove to be more effective and benefit neonates with moderate to severe HIE (Figure 1).

KEY PRECLINICAL GATING ITEMS FOR STEM CELL THERAPY FOR HIE

Academicians, industry partners, and regulators, which include both the National Institutes of Health (NIH) and the U.S. Food and Drug Administration (FDA), have jointly created Stem cell Therapeutics as an Emerging Paradigm for Stroke (STEPS). Together, they have provided guidelines to increase the successful outcome

Abbreviations: CP, cerebral palsy; HI, hypoxic-ischemia; HIE, hypoxic-ischemic encephalopathy; MSCs, mesenchymal stem cells; NE, neonatal encephalopathy; PVL, periventricular leukomalacia; STEPS, stem cell therapeutics as an emerging paradigm for stroke.

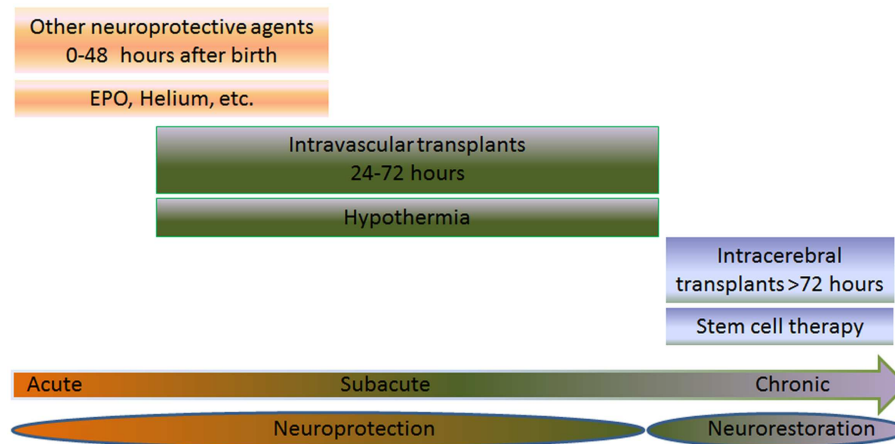


FIGURE 1 | Multiple intended combination therapies are shown for three different groups: acute (0–48 h after birth), subacute (6–72 h after birth), or chronic (>72 h after birth). The joint effort of both cell transplantation therapy and neuroprotective therapies, such as hypothermia, erythropoietin (EPO), and helium are used for treating neonates with HIE. When treating the neonate in the acute or subacute phase, the treatment is referred to as neuroprotection. On the other hand,

treatments aimed at the chronic stage are referred to as neurorestoration. EPO or helium is used to treat the subject during the acute phase, while hypothermia and stem cell therapy is used to treat the subacute stage via intravascular routes. When treating the chronic stage, stem cell therapy is used via intracerebral route, which stimulates the neurorestorative mechanisms. The combination of these therapies may prove to be effective in neonates suffering from HIE.

of cell therapy in stroke patients (24–31). The need for the establishment of a Baby STEPS consortium is necessary for younger patients (32). This would allow a safe and effective translation of cell therapy in neonatal hypoxic-ischemic brain injury. Below, we identify critical gating criteria in conducting translational studies in order to aid and advance the formation of Baby STEPS guidelines.

CLINICALLY RELEVANT MODELS OF HIE

The animal species and strain should be specially considered in HIE modeling. Rodent models such as Vannucci's model of neonatal hypoxic-ischemic brain injury parallel many pathological events that humans endure during neonate HIE (33). Researchers found that in 7-day-old postnatal rats, which undertook ligation of unilateral carotid artery as well as systemic hypoxia, suffered widespread cell death to cerebral cortex, subcortical and periventricular white matter, striatum, and hippocampus ipsilateral to the ligated artery (34). Rats are not the only species used to create the Vannucci model, but mice have also been used (35) with varying pathological outcomes dependent on the mouse strain (36–39).

Another equally important variable that should be controlled in the experimental HIE is the age of the animals. Younger animals have shown to be impervious against hypoxia. A 1–2-day-old postnatal rat needs to be exposed to more severe hypoxia as compared to its 7-day-old counterpart in order to attain efficacious HIE symptoms (40). Another important fact to note is that younger animals experience worse white matter injury than older rats (35). Therefore in HIE modeling, age is a critical factor and is further verified by a focal subcortical cell loss paired with a surge in proliferating oligodendrocyte progenitor cells following HIE in young neonates, but rather modest in older models (41–45). Age-related changes following HIE need to become standardized in

order to better evaluate the therapeutic benefits of experimental treatments.

Gender should also be taken into consideration for models, in that HIE-induced female neonates displayed a much smaller infarct volume and improved sensorimotor task than their male counterparts after perinatal hypoxic-ischemic brain injury and treatment with erythropoietin (46). A possible explanation behind the differences between female and male neonatal infarct sizes and improved neurological behavior has become increasingly clear. It is known that gender differences in injury to the brain are not merely a result of hormonal influence (47), but the properties of individual cells (48). For instance, male and female cells display differential gene expression even when no hormonal influences are apparent (49), and brain cells show phenotypic differences that are gender dependent but independent of gonadal phenotype (50). Moreover, gender modulates responsiveness to recombinant erythropoietin (Epo) (51). Additionally, Epo receptor (EpoR) alleles, EpoRA1, and EpoRA10, have displayed a significantly higher frequency in females when compared to males (52). Epo administration is known to produce significant long-term neuroprotective benefit on the developing brain (46). This suggests that Epo has a gender preference with neonatal benefit in females, whose mechanisms must be further investigated. Together with other studies demonstrating that gender similarly affects injury models (53, 54), these studies suggest that gender should be carefully considered in experimental HIE.

The approximation of the clinical pathology of HIE is a crucial goal in standardizing the animal models because a model that better mimics a human condition would allow for better evaluations of possible treatments in human patients. Rats along with many other species, which include non-human primate, sheep, lamb, puppy, piglet, and rabbit, have been employed as models to closely

resemble some HIE pathological aspects in humans (33, 55–60). Unfortunately, because of the expense in using large animal models, it has deterred research with these clinically relevant models. The piglet model is a good model for research regarding treatment plans for neonates, as it closely resembles the weight and size of a newborn infant. This piglet model also reveals new treatment variables, in that phosphorylated metabolites are temperature-sensitive and that the more severe the energy depletion the worse the secondary energy failure, exacerbating neuronal death (61, 62). These findings suggest the need to control temperature and maintain brain injury in experimental models using therapeutic strategies.

CLINICALLY RELEVANT EXPERIMENTAL PARADIGMS

Lab-to-clinic translatable functional tests need to be developed to better assess the pathological improvements of experimental interventions for short and long-term outcomes that are species specific and mimic the human condition. Despite efforts of investigators to control many variables in experimental HIE models, characterizing the phenotype of encephalopathy in neonates has proven to be elusive. As opposed to adults, neonatal encephalopathy is prevented from implementation of timely interventions as there is a scarcity in studies determining the optimal supra-acute to chronic therapeutic window in laboratory models, thus presenting a major barrier to translating experimental treatments to clinical applications. Acknowledging this research gap is pivotal when designing therapeutic intervention studies for future clinical applications in neonates. In order to create well-designed translational studies, it is critical to optimize the dosage, delivery route, and timing of stem cell transplantation within applicable clinical parameters. Treating the laboratory as the clinical setting for cell therapy in HIE will enhance the translational potential of the stem cell product. In order to prevent any potential microembolism, the minimum therapeutic cell dosage must be determined. Finding minimally invasive procedures for cell delivery could prevent exacerbation of the already injured brain. For timing of cell delivery, consideration should be given to the neuroprotective phase (< 1 day of injury) and the neurorestorative phase (> 1 day after injury) (63, 64). While most transplant studies have examined a single bolus injection of stem cells, we recognize that HIE is a disorder that involves progressive neuronal cell loss even days after HI. As a result, multiple treatments of stem cells and adjunct neuroprotective therapies are more likely to have not only neuroprotective but also neurorestorative effects. This would provide a continual targeted regime to prevent cell death in the hopes to improve neurodevelopmental outcome. For example, two MSC injections at 3 and 10 days after neonatal hypoxia-ischemia (HI) markedly improved sensorimotor function 4 weeks after the insult (65). This MSC-3 + 10 treatment was more powerful in improving functional outcome and in reducing gray and white matter loss than a single MSC injection at 3 days after HI (65).

WELL-DEFINED PHENOTYPIC MARKERS OF DONOR STEM CELLS

A detailed description of the distinguishable phenotypic features of stem cells is an integral component of understanding the biology and potential of stem cells (66–74). To determine the safety and effectiveness of stem cells that are to be transplanted in HIE,

it is important to know their identity. This will also give way to understanding the mechanisms allowing the functional amelioration that takes place after transplantation. That different cell types in the brain undergo degeneration has prompted the notion that brain repair consists of several mechanisms facilitating various types of cells working together with the trophic, neurogenic, vasculogenic, and other by-stander effects of the transplanted stem cells (75). Accordingly, such multi-pronged cellular repair process necessitates the need to determine the source of the stem cells to aid in realizing the therapeutic effects and mechanisms of action associated with cell therapy. Another aspect to consider is the ability of the cells to be shipped frozen and thawed for transplantation at the clinic, which is important for neonatal diseases which may benefit from early intervention. Moreover, using autologous stem cells can be of an advantage due to their ability to bypass graft rejection along with its side effects. For example, in a study where intravenous injection of autologous cord blood was made, the results showed that they were safe in CP children (76). In parallel, placental tissue obtained during prenatal chorionic villous sampling or at delivery can be a good source of autologous stem cells which can be transplanted during the last month of gestation or the first few months after delivery if neurodegeneration is detected in the baby (77).

FUNCTIONAL OUTCOMES IN CELL TRANSPLANTATION IN HIE

To assess the safety and efficacy of the treatment group in HIE, specific behavioral and histological procedures are frequently used. These assays can determine motor and cognitive amelioration as well as provide insight to the biochemical processes that are undergoing in the brain (68, 78, 79). However, to value the functional improvement after cell transplantation in animals, a close estimate of the HIE clinical symptoms needs to be shown in these preclinical models (80–83). In addition, monitoring the safety and efficacy of stem cells over time will allow a better assessment of the effects of cell therapy (84). This becomes an obstacle for neonatal HIE due to the spontaneous amelioration seen in the development and maturation phases of the neonatal animal (85) and in pediatric patients (86). More sensitive functional outcomes may be needed to delineate spontaneous recovery from true therapeutic benefits of cell therapy; for example, to examine the grafted cells and host HIE microscopically, specific markers for the trophic factor effect, immunomodulatory response, neurogenesis, vasculogenesis, angiogenesis, and synaptogenesis, as well as inflammation, tumorigenesis, or ectopic tissue formation may be used (87–89). This approach allows a better understanding of the cells' mechanism of action and indication of unfavorable side effects.

MECHANISMS OF ACTION UNDERLYING CELL THERAPY

Two major modes of action are involved in stem cell-mediated functional recovery in ischemic brain injury: cell replacement and by-stander effect. Cellular and molecular neurorestorative mechanisms include neurogenesis, angiogenesis, synaptogenesis, immunomodulation, and trophic factor secretion (30, 31, 90). Real-time visualization techniques (i.e., magnetic resonance imaging), originally performed in stroke and extended to HIE models (91–93), have allowed, recently, the tracking of the transplant,

as well as the imaging of the host neurorestorative mechanisms (94–100). The neurorestorative mechanism is characterized by transplantation of stem cells that serve as biobridge for the initiation of endogenous repair mechanisms (101). The transplanted stem cells pave the way between the neurogenic niche and injured brain site in order to traffic the migration of host neurogenic cells (101).

OUTSTANDING PRECLINICAL ISSUES NECESSARY FOR CLINICAL TRIALS OF CELL THERAPY IN HIE

Preclinical trials on stem cells in cerebral palsy have been conducted and have shown significant improvement in animal models. However, results have been difficult to replicate in humans since most of the studies have been on acute hypoxic injury models, which are less similar to human cases compared to chronic models. So far, clinical trials on children with cerebral palsy (CP) have suggested that neural progenitor cells (NPC), umbilical cord mononuclear cells, and mesenchymal stem cells (MSC) transplants are safe, and could improve motor and cognitive functions (102). Currently, there are three ongoing registered clinical trials on stem cells and CP on children, and two that have recently been completed.

Two of these clinical trials are currently being conducted in the United States, with the aim of testing the safety and efficacy of autologous cord blood cells. The primary outcomes should be expected by the year 2015. Although patient recruitment is not open, China intends to test the efficacy of umbilical cord MSC versus rehabilitation treatments on children with cerebral palsy. In Iran, two clinical trials have already been completed. These clinical trials studied the efficacy of multiple intrathecal bone marrow-derived CD133 cell transplantation in children with cerebral palsy. Results are not published yet (103).

It is imperative to set the parameters defining safety and efficacy of stem cell therapy in neonatal HIE clinical trials. In the United States, the Medical College of Georgia and Duke University are assessing the safety and efficacy of umbilical cord blood transplants in CP pediatric patients. The long-term efficacy of intravenous transplantation of autologous cord blood in CP children remains to be determined (76, 104). So far, autologous bone marrow-derived MSCs have also been found to be safe when transplanted, but it was only on one CP patient (105). Autologous stem cell sources have been preferred largely due to their safety profile, but cell types that have already committed to a particular lineage or niche in the brain also have the potential to be used as donor cells. In order to implement cell therapy in neonatal ischemic-injury patients, there should be sufficient evidence of safety, efficacy, and mechanism of action of the stem cells in animal models. Moreover, it has been difficult to obtain a projection of the neurologic outcomes of cell therapy in neonatal hypoxic-ischemic injury. The current reports on clinical improvement after cell therapy in children with CP or HIE should not interfere with the need for sufficient preclinical studies for the advancement of clinical trials. The guidelines mentioned in the previous sections on the Baby STEPS may also be implemented to other potential therapies for neonatal hypoxic-ischemic injury (106–109) and should be used along with the current pediatric stroke recommendations in research and treatment interventions (110–113).

In the end, while autologous stem cells have shown to be safe and effective as a possible treatment for HIE, more preclinical studies, paralleled by carefully designed limited clinical trials should be conducted before moving into larger studies.

CELL THERAPY AND ADJUNCTIVE TREATMENT WITH HYPOTHERMIA

The current therapies for HIE attempt to interrupt the pathways activated by HIE. In neonates with HIE, the results have not been promising in regards to preventing the neuronal loss (114, 115). In research models of HIE, it has been shown that hypothermia reduces the release of glutamate (116), reduces the secondary energy failure (21, 61, 116–118), normalizes protein synthesis (119), and reduces the injury by free radicals (115). Small trials of hypothermia conducted on human neonates (117, 118) showed promising results, while three large trials showed improvement in the neurodevelopment in neonates with mild to moderate HIE, but showed no improvement in neonates with severe HIE (20–22). There is evidence that hypothermia may exert neuroprotection by reducing the neurodevelopmental disability at 18 months of age in newborns with either moderate or severe HIE (120). Neuroprotective approaches could reduce the initial tissue damage, however, cell therapy will still be required to repair the already damaged regions of the brain. This approach can then be implemented on neonates with moderate to severe HIE.

In normal physiological conditions, the endoplasmic reticulum (ER) and mitochondria sequester calcium when intracellular levels increase (121). Calcium enters the cell through voltage-sensitive calcium channels and agonist-operated calcium channels, which are activated by glutamate, *N*-methyl-D-aspartate (NMDA) and kainate, and quisqualate (K/Q) (121). In the ischemic cascade, the increase in hydrogen displaces calcium from intracellular proteins and intracellular calcium levels increase eventually leading to mitochondrial dysfunction (121). In addition, calcium activates intracellular proteases and depolarization occurs in the cell membrane, releasing a large number of excitatory neurotransmitters such as glutamate (121). This activates NMDA receptors persistently, causing intracellular hyperosmolarity in the post-synaptic cell eventually leading to neuronal death (121). Moreover, the ongoing sodium influx inhibits the normal magnesium blockade on NMDA receptors (121). Hypothermia significantly reduces extracellular levels of excitatory neurotransmitters (121). The release of these neurotransmitters is temperature dependent and even mild levels of hypothermia exert an inhibitory effect (121). Hypothermia promotes the survival of neurons through an interaction on glycine since NMDA receptors require the presence of glycine to be activated (121). Hypothermia significantly decreases brain glycine levels after ischemia, thus decreasing hyperexcitability by glutamate (121).

Hypothermia exerts neuroprotection in HIE against aberrant stages of region-specific brain maturation (122), blood–brain barrier (BBB) impairment (123), and apoptosis due to mitochondrial dysfunction (124). Because hypothermia is most efficacious within the first 6 h of life for the infant with moderate to severe HIE (125–127), the patient may benefit even more with the use of a combinatorial therapies (128, 129). For example, using erythropoietin and helium, both which are in clinical trials (130–132),

should be considered for in combination therapy. The treatment considerations for hypothermia in addition to combining other therapies are based on the evolving pathophysiology of neonatal brain injury, discussed by Ferriero et al. (133, 134). Using a combination of therapies may be more beneficial to tackle the activated cell death pathways; moreover, detecting it early in at-risk newborns may help prevent or reduce the disabilities following neonatal brain injury (133, 134).

As discussed above, accumulating experimental data have indicated the mobilization of bone marrow-derived stem cells, such as MSCs, in brain plasticity and therapy of HIE to the affected area (135). In the clinic, MSCs can be obtained from umbilical cord blood, adipose tissue, amniotic fluid/tissue, or menstrual blood (136). As alluded earlier, autologous MSCs may be the preferred stem cells to avoid adverse effects associated with graft rejection, but allogeneic MSCs may also be equally safe and effective because of their immature immune system, as well as their capacity to secrete anti-inflammatory factor (136). MSCs are capable of differentiation into variety of phenotype cells (130, 137) and have been demonstrated to exert a therapeutic benefit against brain injury (125). However, little is known regarding MSC treatment for HIE, especially in combination with hypothermia.

The observation that seizure onset beyond the first 12 h of life is not only common in newborns with HIE (138), but also is associated with severe brain injury (56), advances the notion of a critical relationship between the onset of neonatal seizure and initiation of the therapy. Accordingly, any treatment regimen, including hypothermia, is likely to exert benefit if initiated within 6 h after hypoxic-ischemic injury and continuing over the next 12 h or even beyond (i.e., for 72 h) (138). The mechanism underlying hypothermia remains elusive, but may include its capacity to reduce oxidative stress, energy deficit, and inflammation (139). Because of the dismal prognosis of infants with HIE, clinical enthusiasm for a novel treatment is understandable (140). Based on preclinical studies, accumulating evidence suggests that in order to treat more effectively neonatal HIE, and many other neurodegenerative diseases, combination therapy can be of great help. As ischemic brain injuries have two separate cascades of events, one immediately after injury and one persisting even weeks after, it is important to use combination therapy which can tackle both events at different times. We highlight here that hypothermia could be a great neuroprotective method implemented early in HIE, while stem cells could have a better neurorestorative approach, especially during the chronic stage of the disease, which starts days after (i.e., 72 h after birth).

As mentioned previously, combination therapy may be the best approach to treat neonatal HIE, especially when a definite therapeutic time frame has not been fully established. The use of other possible neuroprotective strategies has been studied and is believed to enhance the therapeutic effects of hypothermia by targeting different therapeutic windows and stages of HIE. Oxygen free radicals are usually elevated after hypoxic-ischemic injury. The use of antioxidants like superoxide dismutase, combined with polyethylene glycol to facilitate infiltration across the blood-brain barrier, can degrade reactive oxygen species to ameliorate the negative effects of hypoxia. Nonetheless, neuroprotection in newborn animals has only been seen when administered prior to

injury. Xanthine oxidase inhibitors, like allopurinol and oxypurinol, have also shown to reduce concentrations of free radicals in infants when administered early during the recovery phase, while administration of lazeroids appears to inhibit iron-dependent lipid peroxidation in immature models of hypoxic-ischemic brain damage (141, 142). Epo appears to have a longer therapeutic window (24–48 h after delivery) compared to hypothermia. Administration of Epo leads to a reduction in white matter injury, free radical production, and glutamate cytotoxicity in neonates after a hypoxic event by increasing the system XC-expression (130, 142, 143). System XC exchanges 1 mol of cystine for 1 mol of glutamate leading to increased cellular viability (143). The therapeutic effects of hypothermia and IV melatonin in perinatal asphyxia piglets model have been reported (144). Glutamate antagonists such as MK-801 have been shown to reduce brain damage after hypoxic-ischemia in neonatal animal models (141, 144). The accumulation of calcium in the cytosol is also characteristic of hypoxic-ischemia. Flunarizine, a Ca²⁺ channel blocker, has shown to have a neuroprotective effects when administered prior to injury in fetus and newborn animals (141). Magnesium sulfate has also shown to be a potential treatment to reduce newborn brain injury (in rats) by blocking the neuronal influx of Ca²⁺ within the ion channel, while it could also reduce the incidence of moderate to severe CP (141, 144). However, the use of Ca²⁺ blockers has been linked to adverse cardiovascular effects on infants, contraindicating their use. It has been observed that neuronal loss can be reduced through the administration of NOS inhibitors in immature rats; however, further studies need to be conducted in order to prove its effectiveness in other animal models (141). Neuroprotective effects after the administration of noble gases like xenon, argon, and helium 2 h post hypoxic injury in 7-day-old SD rat has been reported. Although helium seems to be neuroprotective only in mild hypoxic-ischemic injury, argon and xenon showed neuroprotection in severe hypoxic animal models by increasing Bcl-2 and Bcl-xL, as well as reduction of infarct size and long-term neurocognition (144, 145). There are still many potential neuroprotective strategies to be studied including protection by neural stem cells and the ependymal lining of the ventricles, among others, which will similarly require further investigations for HIE applications.

The use of delta opioid agonists may resemble certain physiological correlation of hibernation, including hypothermia (71), which may involve direct opioid receptor activation, as well as non-opioid mechanisms (71, 146, 147). Interestingly, delta opioids may regulate neural stem and progenitor cell proliferation and differentiation (148), and may even enhance cell-based therapeutics in *in vitro* and *in vivo* disease models (149). Our recent study (150) revealed that moderate hypothermia is efficacious in an *in vitro* model of hypoxic-ischemic injury, which was enhanced by MSC treatment. We also showed that the delta opioid system, along with other non-opioid neuroprotective processes, primarily contributes to the observed neuroprotection in HIE. Stem cell therapy using MSCs significantly improved the therapeutic outcome of moderate hypothermia. Primary rat neurons were exposed to oxygen-glucose deprivation (OGD) condition, a model of hypoxic-ischemic injury, then incubated at 25°C (severe hypothermia), 34°C (moderate hypothermia), and 37°C (normothermia) with or without subsequent co-culture with MSCs. Combination

treatment of moderate hypothermia and MSCs proved to be the optimal condition for preserving cell survival and mitochondrial activity after OGD exposure. Pharmacologic induction of hypothermia in human embryonic kidney cells (HEK293) via treatment with delta opioid peptide (DADLE) resembled moderate hypothermia's attenuation of OGD-mediated cell alterations, which were much more pronounced in HEK293 cells overexpressing the delta opioid receptor. Further, the addition of DADLE to 34°C hypothermia and stem cell treatment in primary rat neurons showed synergistic neuroprotective effects against OGD which were significantly more robust than the dual combination of moderate hypothermia and MSCs, and were significantly reduced, but not completely abolished, by the opioid receptor antagonist naltrrexone altogether implicating a ligand–receptor mechanism of neuroprotection. Investigations into other therapeutic signaling pathways revealed growth factor upregulation (i.e., GDNF) and anti-apoptotic function accompanying the observed therapeutic benefits. These results support combination therapy of hypothermia and stem cells for hypoxic-ischemic injury, which may have direct impact on current clinical trials using stand-alone hypothermia or stem cells for treating neonatal hypoxic-ischemic brain injury.

The use of hypothermia as a treatment strategy is not limited to neonates. In adults, hypothermia has been regarded as a therapeutic strategy to improve the patient's survival and reduce neurologic injury after cardiac arrest (151). Small clinical trials provide evidence of hypothermia as a potential treatment for traumatic brain injury (TBI) as it significantly reduced rates of death, vegetative state, and long-term disability (152). Although not regarded as a treatment strategy for spinal cord injury (SCI), the control of pressure around the injured spinal cord along with the improved behavioral outcome in animal studies demonstrates the potential of systemic hypothermia as a method of treating acute SCI (153). When used in combination with recombinant tissue plasminogen activator, animal data also show a reduction in brain hemorrhage and blood–brain barrier disruption, indicating the synergistic potential of hypothermia in stroke (154).

In addition, the use of stem cells as a therapeutic strategy in adults has obtained much attention due to the substantial beneficial data gathered in animal and clinical studies. For example, specific disease-relevant neurons can be obtained from induced pluripotent stem cells including dopaminergic neurons for Parkinson's disease (PD), hippocampal and cholinergic neurons for Alzheimer's disease, motor neurons for amyotrophic lateral sclerosis, and inhibitory interneurons for schizophrenia (155). Embryonic stem cells can be made to also differentiate into dopaminergic neurons and have been shown to ameliorate the behavioral deficit in animal models of PD (156). Embryonic neural stem cells are also considered in demyelinating diseases, such as multiple sclerosis, to replace glial cells that have been lost (156). In addition, the use of neural stem cells in Huntington's disease has been evident (156).

SYNOPSIS

Stem cell therapy has the potential to become a treatment method for neonatal hypoxic-ischemic brain injury, but it needs to be first implemented in the clinic on patients with neonatal hypoxic-ischemic brain injury. This will require further translational

research studies so that stem cell therapy can be fully implemented. Stem cell therapy can also benefit from the ongoing trials of stem cell therapy in adult stroke due to the similarities in pathology. Neonatal hypoxic-ischemic injury, however, has distinct symptoms from adult stroke (157–159) that will demand modifications to the translational approach before it can be applied to the clinic. Combining both hypothermia and stem cell therapy may further improve the results of cell therapy in neonatal hypoxic-ischemic injury. The results from using this combinatorial approach can be then measured by behavioral and histological assays. In addition, using biomarkers to monitor the transplanted cells in the patient over time will contribute to exposing the long-term effects of this combinatorial therapy.

Currently, the use of stem cells for neonatal hypoxic-ischemic brain injury is still in its experimental phase. Although clinical trials are scheduled to be conducted using autologous umbilical cord blood cells on CP children, there still needs to be a sufficient amount of data demonstrating the safety and efficacy before transplantation therapy can be used in other neonatal diseases. It is a priority to obtain standardized experimental models with quantitative functional endpoints and predictive clinical outcomes so that translational research can be implemented. In addition, it is important to address and modify the following for an effective future use of stem cell therapy: the route of delivery, cell dose, and timing of transplantation after diagnosis of neonatal brain injury. It should also be noted that because it is difficult to detect encephalopathy in premature babies due to the lack of advanced imaging devices and sufficient studies, initial cell therapy clinical trials will consist of full term infants. Because it is agreed that HIE involves several cell death pathways, it is expected that more laboratory research on combination therapies will be conducted in the future. Specifically, incorporating clinical stage therapies, such as hypothermia and other neuroprotective strategies, with stem cell transplantation therapy will be of future focus. Moreover, the safety and efficacy of these combinatorial strategies for neonatal hypoxic-ischemic brain injury can be maximized by following pertinent translational research guidelines [e.g., Ref. (32)].

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Conflict of Interest Statement: Cesar V. Borlongan holds patents and has pending patent applications in stem cell biology and therapeutic applications.

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